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A New Integrative Conjugative Element Occurs in *Mycoplasma agalactiae* as Chromosomal and Free Circular Forms

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An integrative conjugative element, ICEA, was characterized in *Mycoplasma agalactiae* strain 5632, in which it occurs as multiple chromosomal copies and as a free circular form. The distribution of ICEA sequences in *M. agalactiae* strains and their occurrence in *Mycoplasma bovis* suggest the spreading of the element within or between species.

Integrative conjugative elements (ICEs) are mobile, modular sequences that are present in many bacterial species and that spread from donor to recipient by conjugation (4, 19). In mollicutes, such an element has not been thoroughly described except for the ICEF of the human-infecting *Mycoplasma fermentans* strain PG18, in which it occurs in two versions, ICEF-I (1 copy) and ICEF-II (3 copies), and produces a circular form after excision from its locus (6). This mycoplasma belongs to the hominis phylogenic group, along with *Mycoplasma agalactiae* and *Mycoplasma bovis* (15), which are ovine-caprine and bovine pathogens, respectively.

Sequence analysis of an ICE in *Mycoplasma agalactiae*. Recently, an *M. bovis* DNA fragment related to a portion of ICEF was detected in *M. agalactiae* strain 5632 by Southern blot analysis (10). Since strain 5632 is currently sequenced and partially assembled, we searched for ICE-related sequences and found a contiguous 27-kb genomic region, $ICEA_{5632}$-I, containing 12 coding sequences (CDSs) homologous to ICEF open reading frames (ORFs), and further designated by us with the same numbers as Calcutt et al. (6) (Fig. 1). Sequence manipulations and alignments were performed using the Artemis program (18) and the Infobiogen website (http://www.infobiogen.fr).

Features of $ICEA_{5632}$-I are presented in Fig. 1A and Table 1. Marked differences from *M. fermentans* (Fig. 1B) were observed, consistent with module rearrangements and gene decay. More specifically, $ICEA_{5632}$-I contains 8 CDSs with no homology to ICEF ORFs and lacks 10 ICEF ORF homologs. In $ICEA_{5632}$-I, CDS11, -12, and -14, which display 34%, 31%, and 44% similarity with ICEF ORF11, -12, and -14, respectively, are located differently in the two elements. $ICEA_{5632}$-I contains four large intergenic segments, mostly in regions that differ significantly from ICEF, and three CDSs (CDS-F, -G, and -H) clustered in opposite orientation relative to the others. The $ICEA_{5632}$-I CDS products were systematically analyzed with the PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), Pfam search (http://www.sanger.ac.uk/Software/Pfam/), ScanProsite (http://www.expasy.org/tools/scanprosite/), PHD (17), and PSORT (http://psort.nibb.ac.jp/) programs, showing some relations with conjugation, plasmid, or phage proteins (Table 1). CDS5 is homologous to TraG, a conjugation protein that couples the relaxosome to the translocation apparatus (5, 9). CDS17 is homologous to TraE, a membrane-bound ATP-GTP binding protein essential for DNA transport across the conjugation channel (7, 16). CDS12 is weakly similar to ICEF ORF12 and contains a single-stranded DNA binding domain which may prevent the transferred DNA from degrading in the recipient cell. CDSA is related to plasmid-encoded anti-restriction protein ArdC (3) and primase TraC (11), although it lacks the primase active-site motif AGYATA (20), suggesting an anti-restriction or atypical primase function during or after $ICEA_{5632}$-I transfer. CDSG is related to MinD/ParA proteins involved in chromosome or plasmid DNA partitioning (13) and could control the replication of an ICEA extrachromosomal form, or could represent the nonfunctional remains of a co-resident plasmid. Several CDS products predicted to be membrane associated or to contain transmembrane domains could be involved in a mating pore formation. CDSH is homologous to several bacterial DNA methyltransferases and could control $ICEA_{5632}$-I survival and propagation through various hosts.

Presence of a circular extrachromosomal $ICEA_{5632}$-I form and identification of the termini. The ICEF termini contain inverted repeats that are linked together by a short coupling sequence following the element excision to form an extrachromosomal circular intermediate (6). Three interspersed nucleotide motifs (designated I, II, and III) (Fig. 2A) surround the integrated $ICEA_{5632}$-I and are composed of a direct and an inverted repeat of 9 and 4 bp, respectively, a pattern similar but not identical to that of ICEF. An $ICEA_{5632}$-I circular intermediate was detected by standard PCRs using 5632 purified genomic DNA as a template and primers located at each end of the element (Fig. 2B and C; Table 2). Direct sequencing of the PCR fragment generated with primers Right2 and Left1 (Fig. 2C, lane 1) identified the 4-bp inverted repeat of motif II,

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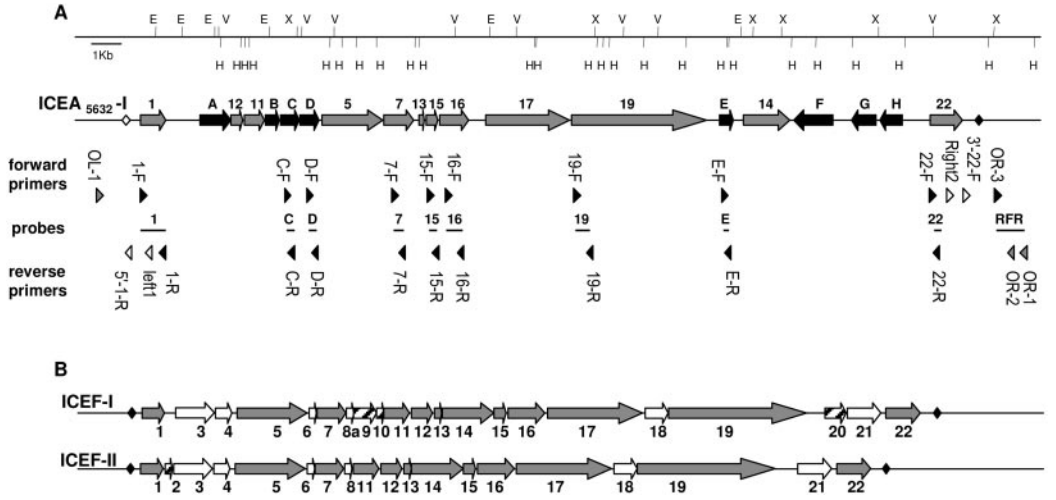

FIG. 1. Genetic organization of ICEs characterized in *M. agalactiae* and *M. fermentans*. (A) Restriction map and genetic organization of the $ICEA_{5632}$-I locus of *M. agalactiae* strain 5632. E, EcoRI; V, EcoRV; X, XbaI; H, HindIII. Diamonds indicate the ICE termini; gray arrows, ORFs found in both $ICEA_{5632}$-I and ICEF; black arrows, CDSs found only in $ICEA_{5632}$-I. The locations of the specific probes (horizontal bars) and of the PCR primers (filled or open arrowheads) used in this study are indicated below the $ICEA_{5632}$-I locus representation. (B) ICEF versions previously described for *M. fermentans* strain PG18. White arrows, ICEF-specific CDSs; hatched arrows, locus-variable ICEF-specific CDSs.

separated by a 7-bp heterogeneous coupling sequence with ambiguous positions, whereas the rest of the sequencing reaction gave an unambiguous read (Fig. 2B). It is unlikely that this heterogeneity is due to PCR and/or sequencing artifacts, since identical results were obtained on both strands. In view of the fact that several $ICEA_{5632}$ copies occur in the genome (see below), sequence ambiguities rather suggest the presence in the PCR template of $ICEA_{5632}$ circular intermediates generated by the excision of distinct copies, which all contain a coupling sequence of 7 bp. The Right2/Left1 amplicon was also cloned into *Escherichia coli*, and a randomly selected recombinant plasmid was shown after sequencing to harbor a unique 7-bp coupling sequence, CGTAATT, that matches the direct-repeat sequence of motif II (CGTAATTTT) of $ICEA_{5632}$-I. Sequencing of a PCR product obtained with primers located outside of the integrated $ICEA_{5632}$-I (Fig. 2D; Table 2) revealed that excision of $ICEA_{5632}$-I connects the left and right regions that flanked the element, resulting in a unique 9-bp sequence identical to the motif II direct repeat. It is noteworthy that while the full 9-bp sequence remains on the chromosomal locus following $ICEA_{5632}$-I excision, only 7 bp was incorporated into the extrachromosomal form of the element. This might correspond to a specific excision mechanism that results in the deletion of two T bases during the juxtaposition of the termini on the circular form. These data suggest that the excision-integration of $ICEA_{5632}$ and ICEF, if comparable, might not be identical. In both systems the gene(s) supporting this function has yet to be identified.

Strain and species distribution of $ICEA_{5632}$-I sequences. Two previously described *M. agalactiae* and *M. bovis* strain collections (10) were analyzed by Southern blotting using PCR probes labeled with digoxigenin (Dig)-11-dUTP (Roche) and primer pairs located in $ICEA_{5632}$-I genes (Fig. 1; Table 2) with or without ICEF counterparts. Only 3 out of 32 *M. agalactiae* strains (including strain 5632) reacted with all probes, while 38 out of 56 *M. bovis* strains reacted with all but CDSE and CDS22. There was no correlation between the presence of ICEs and the geographical origin of the isolate or the year of isolation. *M. agalactiae*, *M. bovis*, and *M. fermentans* ICEs may have been inherited from a common ancestor and undergone sequence divergence over time, resulting in partial or total sequence losses. Alternatively, ICEs may have spread by lateral transfer during coinfections of ruminants as a common niche (2), giving rise to new subpopulations in which the elements may further multiply and evolve. In this respect, it is noteworthy that *M. fermentans*, although known as a human-infecting mycoplasma, was recently isolated from a small ruminant (14).

ICEs appear to be absent from the mollicutes sequenced so far, with the exception of *Mycoplasma hyopneumoniae* strains 232 (12) and 7448 (22) and *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343 (GenBank accession number CP000123), in which CDS clusters partially homologous to ICEF have been annotated (locus tags, mhp521 to mhp534, MHP7448_412 to -424, and MCAP_0554 to -0571). Characterization of these putative elements has not been documented so far. Sequences encoding TraE homologs were also identified in *Mycoplasma pulmonis* (NP_326214), *Mycoplasma mycoides* subsp. *mycoides* SC (NP_975194), and *M. hyopneumoniae* (YP_116041 and YP_287807) genomes and in uncharacterized extrachromosomal (1, 8) or plasmid (8) DNA from *Spiroplasma kunkelii*, but evidence for the presence of functional ICEs in these species is still lacking.

Number of $ICEA_{5632}$-I copies in the *M. agalactiae* strain 5632 genome. Southern hybridization of Dig-labeled probes for $ICEA_{5632}$-I CDS1 or CDS22 (Fig. 1; Table 2) suggested the presence of at least 3 ICEA chromosomal copies in *M. agalactiae* 5632 (Fig. 3A and B). Furthermore, subsequent hybridization with probe RFR, located within a unique region that flanks $ICEA_{5632}$-I, ruled out DNA rearrangements as a basis for the patterns observed (Fig. 3C). Whether these copies differ from $ICEA_{5632}$-I (except for the common presence of CDS1 and -22) is not known. Restriction fragments that hy-

TABLE 1. $ICEA_{5632}$-I homology searches and relevant features of potential products

CDS	Size (aa)	MW[a]	Best Blastp hit	E value	Organism[b]	Accession no.	Result of:					Proposed function
							psi-blast[c]	Pfam search	Prosite search	PHD[d]	PSORT[e]	
ICEF-homologous putative coding sequences												
CDS1	264	31,324	ICEF II A ORF1 (239 aa)	$2 \cdot 10^{-10}$	*M. fermentans*	AAN85234.1	Q8GCM7 (ICEF ORF1)			2	C	Mating protein
CDS5	670	77,585	ICEF II A ORF5 (739 aa)	$1 \cdot 10^{-159}$	*M. fermentans*	AAN85238.1	Q97HP1 (possible plasmid transfer factor)	TraG	PS00017 (ATP/GTP binding site motif A)	2	M	Coupling protein
CDS7	326	37,082	ICEF I A ORF7 (315 aa)	$1 \cdot 10^{-18}$	*M. fermentans*	AAN85217.1	Q8GCL4 (ICEF ORF7)			1	M	Mating protein
CDS11	221	26,158	ICEF I A ORF11 (238 aa)	0.52	*M. fermentans*	AAN85221.1	None				C	
CDS12	134	15,940	ICEF II ORF12 (183 aa)	0.11	*M. fermentans*	AAN85266.1	Q8GCN6 (single-stranded DNA binding protein)		PS50935 (single-strand binding domain)		C	Single-strand DNA protection
CDS13	84	9,956	ICEF II ORF13 (84 aa)	$1.3 \cdot 10^{-2}$	*M. fermentans*	AAN85267.1						
CDS14	525	61,854	P57 lipoprotein (ICEF II ORF14 [522 aa])	$6 \cdot 10^{-39}$	*M. fermentans*	AAN85271.1	Q8GCK7 (P57 lipoprotein)		PS00013 (lipoprotein lipid attachment site)		M (out)	Stabilization of mating protein
CDS15	121	13,304	ICEF II ORF15 (93 aa)	$3 \cdot 10^{-7}$	*M. fermentans*	DAA01192.1	Q8G8Q8 (ICEF ORF15)			2	M	Mating protein
CDS16	357	39,644	ICEF I A ORF16 (396 aa)	$9 \cdot 10^{-25}$	*M. fermentans*	AAN85226.1	Q8GCN4 (ICEF ORF16)	GP38 (potential)		9	M	Mating/chaperone
CDS17	928	107,956	TraE/TrsE NTPase (ICEF II ORF17 [937 aa])	0.0	*M. fermentans*	AAN85227.1	Q8GCN3 (TraE/TrsE family nucleoside triphosphatase)		PS00017 (ATP/GTP binding site motif A)	2	M	DNA transport
CDS19	1,517	175,201	ICEF II A ORF19 (1,409 aa)	$4 \cdot 10^{-57}$	*M. fermentans*	AAN85241.1	Q8GCK1 (ICEF ORF19 [fragment])	Phage_Capsid (potential)		1	M	
CDS22	378	45,251	ICEF I A ORF22 (388 aa)	$9 \cdot 10^{-68}$	*M. fermentans*	AAN85231.1	Q8GCN0 (ICEF ORF22)				C	
Putative coding sequences not homologous to ICEF												
CDSA	346	40,510	Hypothetical protein PCP36	$2 \cdot 10^{-21}$	*Clostridium perfringens*	NP_150029.1	P27190 (DNA primase *traC*), Q6I6B2 (ArdC protein)				C	DNA primase/anti-restriction
CDSB	150	17,310	P75 protein	0.13	*Mycoplasma hominis*	CAB62240.1	None				C	
CDSC	186	22,160	None				None				C	
CDSD	217	25,860	Hypothetical protein MYPE9110	0.009	*Mycoplasma penetrans*	NP_758298.1	None				C	
CDSE	150	17,648	Putative prophage protein	$3 \cdot 10^{-65}$	*M. mycoides* SC	NP_975197.1	P71160 (GepA protein)				C	
CDSF	420	48,849	Hypothetical protein UU367	$4 \cdot 10^{-40}$	*Ureaplasma urealyticum*	NP_078201.1	Q8DZU7 (hypothetical protein SAG1)			1	C	Mating protein
CDSG	279	32,025	Putative plasmid partitioning protein	$3 \cdot 10^{-16}$	*S. kunkelii*	YP_138227.1	Q82YY2 (ATPase, ParA family)	CbiA			C	DNA partition
CDSH	223	25,976	Adenine-specific DNA methyltransferase	$1 \cdot 10^{-101}$	*M. mycoides* SC	NP_975203.1	Q6MU41 (adenine-specific DNA methyltransferase)	MethyltransfD12	PS00092 (N-6 adenine-specific DNA methylase)		C	DNA protection from restriction enzyme digestion

[a] MW, molecular weight.
[b] SC, small colony.
[c] Relevant results from PredictProtein server.
[d] Number of transmembrane domains predicted by the PHD program.
[e] Cellular localization predicted by the PSORT program. C, cytoplasm; M, membrane; out, surface exposed.

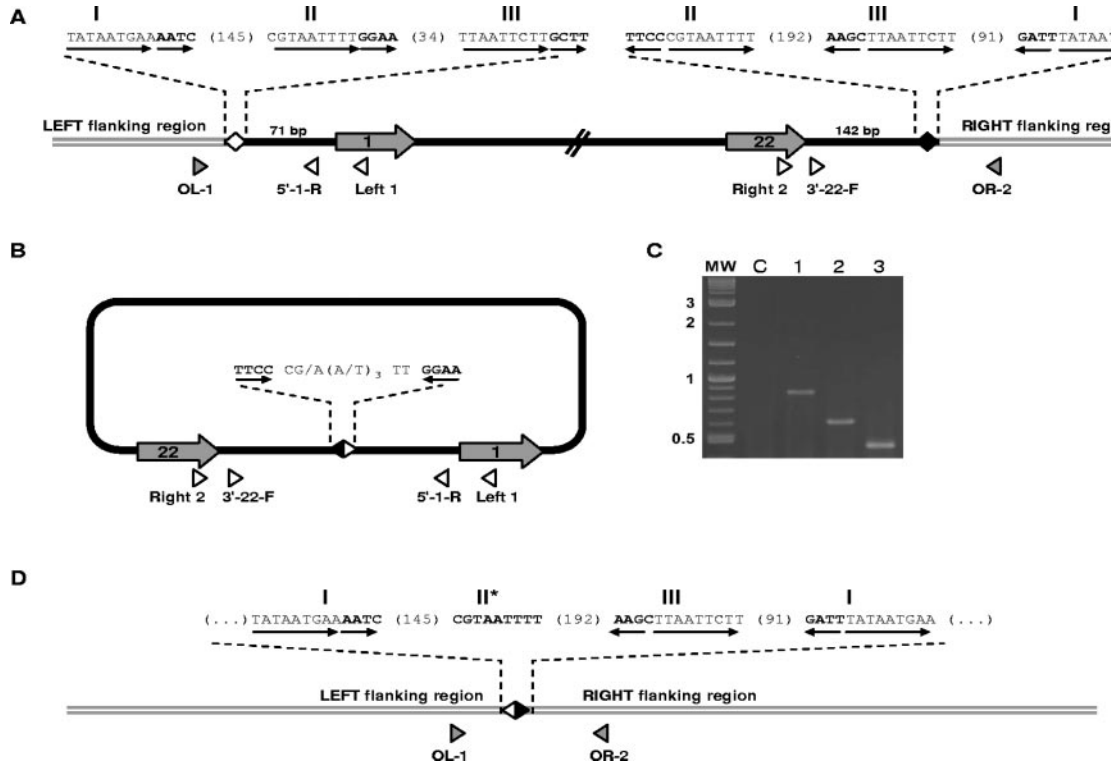

FIG. 2. Occurrence of *M. agalactiae* $ICEA_{5632}$-I as an integrated or free circular form. (A) The integrated $ICEA_{5632}$-I (solid line) and its flanking chromosomal regions (double gray line) are represented along with relevant CDSs (arrows) and PCR primers (arrowheads). Filled and open diamonds, $ICEA_{5632}$-I left and right termini, respectively. Inverted-repeat (boldfaced letters) and direct-repeat sequences of motifs I, II, and III are given above the diagram, with the numbers of nucleotides separating the repeats given in parentheses. (B) Proposed configuration of the $ICEA_{5632}$-I circular form. The black-and-white diamond represents the coupling sequence resulting from the reassociation of the left and right repeats of motif II. (C) $ICEA_{5632}$-I-specific PCR assays analyzed on a 1% agarose gel. MW, molecular weight marker. C, negative control. Primer pairs: Right2/Left1 (lane 1), Right2/5′-1-R (lane 2), 3′-22-F/Left1 (lane 3). (D) Proposed configuration of the chromosomal locus following excision of $ICEA_{5632}$-I. The sequence given above the diagram corresponds to part of the sequence of a PCR product using OL-1 and OR2 as primers and 5632 total DNA as the template. Asterisk indicates the motif II unique sequence resulting from the excision.

TABLE 2. PCR primers used in this study

Name	Sequence
Left1	TAATGGCCAAGAGTTCAAAAGCAA
Right2	TACACAAGTGGTAATGCTGAAACA
5′-1-R	TTCTCCATTTAAACTATCAAAGTTCTATTA
3′-22-F	AAAAGTACTACAAAACAGGGATAAAAA
OR-1	TCCCTCTAATCTTTTTCAAATGCAGA
OR-2	TCATTGGTGCAGGATTTTCA
OR-3	CATCATACTCGCTTGGTGATGTGTTCGC
OL-1	CTCTAATTTCTGCTGGTACTGCCATA
1-F	CCAGTTGTTACACTTGGCTTTTT
1-R	CAAAGGGTTGTTGCTGATCC
C-F	GCCAATAGATTTCAAAGTGAACG
C-R	GCTCTGCCATAGCCATCAAT
D-F	TTCTGCAACAAATTTTGCTTTT
D-R	TCAATCTCTTTCATCCACAAGG
7-F	GCCATCTACAGCGATTCTGTC
7-R	TCAGTTGCTTTTGGGCTTTT
15-F	ATACAGCTGGATTAACAAAACTACAA
15-R	CTAGCTACTCCCGGTGATGG
16-F	CCCAAGCATATTTGCTCACA
16-R	CGCTGCTTCTGGTAATCCAC
19-F	GTGCCAGCATTAGGGAGTTT
19-R	GCCTTCTCTTTCGCAGTTTG
E-F	ATGCTTTGCTTGATGATGGT
E-R	AGCCCTTTCTTGCCTTTTTC
22-F	TGAGACCAGCAAGCTGAAGA
22-R	TCTGTATCAATCTGAATTGCATCAT

bridize with both CDS1 and -22 probes and correspond to the $ICEA_{5632}$-I linked ends in the circular form were not detected by Southern blotting, probably because of the small amount of such target sequences. Because ICEA is apparently repeated in 5632 and because only one copy was assembled from the shotgun sequences, we considered the possibility that $ICEA_{5632}$-I could represent a composite of different elements. Since no large genomic fragment harboring the full locus was available in the sequencing library, we ruled out this hypothesis by several independent experiments. The $ICEA_{5632}$-I locus was validated by: (i) pulsed-field gel electrophoreses on digested 5632 DNA, followed by Southern blot analysis, using probes located inside or outside the element, (ii) restriction and Southern blot analyses of a pulsed-field gel electrophoresis-purified fragment carrying the entire $ICEA_{5632}$-I and its surrounding chromosomal regions, and (iii) various overlapping PCRs with primers anchored outside and/or inside $ICEA_{5632}$-I, followed by restriction analysis of the amplicons. All the results were consistent with the assembled sequence.

The presence of such large repeated elements in organisms that have undergone reductive evolution of their genomes is remarkable and may have been underestimated. ICE structures are modular, and ICEF or $ICEA_{5632}$ could harbor virulence determinants that may influence the pathogenicity of

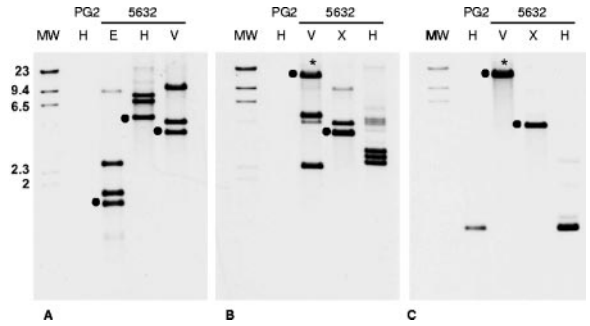

FIG. 3. Southern blot analyses showing the presence of several $ICEA_{5632}$ chromosomal copies in *M. agalactiae* strain 5632. The enzymes used to digest the genomic DNA are indicated on top of each panel: E, EcoRI; V, EcoRV; X, XbaI; H, HindIII. MW, Dig-labeled lambda HindIII DNA. Membranes were hybridized with either a CDS1-specific probe (A) or a CDS22-specific probe (B); the membrane from panel B was stripped and rehybridized with the RFR probe (C). Stars indicate the EcoRV DNA fragments that reacted with both the CDS22 and RFR probes. Dots indicate the fragment displaying the expected size and corresponding to the $ICEA_{5632}$-I locus sequenced in this study.

their hosts. Taken together, these data show that *M. agalactiae* strain 5632 carries a mobile genetic system whose specific excision/integration mechanisms have yet to be elucidated. Finally, the exchange of genetic material in mycoplasmas, which has rarely been documented (21), deserves further investigation. Addressing these questions will provide new insights into the biodiversity, the potential for evolution, and ultimately the virulence of these organisms.

Nucleotide sequence accession number. The $ICEA_{5632}$-I locus sequence (29,942 bp) was deposited in GenBank under accession number CT030003.

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